Isolation by Genetic Labeling of a New Mycobacterial Plasmid, pJAZ38, from Mycobacterium fortuitum

JULIE-ANN GAVIGAN,* JOSÉ ANTONIO AÍNSA, ESTHER PÉREZ, ISABEL OTAL, AND CARLOS MARTÍN

Departamento de Microbiología, Facultad de Medicina, Universidad de Zaragoza, 50009 Zaragoza, Spain

Received 16 January 1997/Accepted 29 April 1997

In a two-step mating experiment with recipient strains of Mycobacterium smegmatis, the Mycobacterium fortuitum cryptic plasmid pJAZ38 was isolated. Plasmid pJAZ38 was genetically labeled by coinintegration formation mediated by the kanamycin-resistant mycobacterial transposon Tn611. The region responsible for replication of pJAZ38 was located and sequenced. This region showed homology with the Mycobacterium avium plasmid pLR7 and the Mycobacterium scrofulaceum plasmid pMSC262, a family of plasmids which have been found to be widespread throughout the mycobacteria. Further experiments showed pJAZ38 to be stably inherited in the absence of selection pressure and compatible with the most commonly used mycobacterial replicon, pAL5000. In contrast to pLR7 and pMSC262, pJAZ38 was able to replicate in M. smegmatis mc^155, making it a useful tool for mycobacterial genetics.

The genus Mycobacterium comprises a wide range of species, from the pathogenic slow growers, such as the causative agents of tuberculosis and leprosy, to fast-growing mycobacteria found in the environment, such as Mycobacterium fortuitum. Traditionally, mycobacterial genetics has not been developed because of the pathogenicity and slow growth rate of Mycobacterium tuberculosis. The global increase in the incidence of tuberculosis, however, has been accompanied by an increase in molecular studies of mycobacteria, and although genetic systems are steadily being developed, there is still a need for more tools in this area.

Several plasmids have been detected in mycobacteria from both environmental organisms and opportunistic pathogens (4, 5, 17). They are especially common in Mycobacterium avium complex isolates recovered from infected humans with pulmonary infections or AIDS and in Mycobacterium scrofulaceum and the M. fortuitum complex of both clinical and environmental isolates (3, 10). Of all of the plasmids isolated, the most extensively studied is the M. fortuitum plasmid pAL5000 (14, 21, 25). This plasmid has been used as the basis for several other mycobacterial vectors (6, 8, 9). The other two mycobacterial plasmids whose replication regions have been defined and sequenced are the M. scrofulaceum plasmid pMSC262 (20) and the M. avium plasmid pLR7 (2). These two replicons were found to show homology in the replication region. Related plasmids have been detected throughout the mycobacteria by hybridization studies with pLR7 as a probe (5).

Several insertion sequences have been isolated from mycobacteria (18), but only one composite transposon, Tn610, has been isolated. This transposon was isolated from M. fortuitum and contains a sulfonamide-resistant determinant and two copies of insertion sequence IS6100 which show homologies with the IS6 family elements. This is the only mycobacterial mobile element whose mechanism of transposition is a replicative process leading to the formation of cointegrates by duplication of one IS element in a direct orientation. Tn611 is a kanamy-

* Corresponding author. Present address: Department of Microbiology, Room 440, University College, Cork, Ireland. Phone: 353 21 90 29 25. Fax: 353 21 90 31 01. E-mail: stdm8053@bureau.ucc.ie.
**RESULTS**

Genetic labeling of a mycobacterial cryptic plasmid. This work resulted from a study of intragenic gene transfer in mycobacteria in which transfer of mercury resistance was detected between *M. fortuitum* 138 and *M. smegmatis* in mating experiments at a frequency of 10^**-2** per recipient cell (our unpublished results). Plasmid DNA preparations with various previously described protocols (11, 12) did not result in the detection of the presence of plasmids in agarose gels, although the presence of three plasmids had previously been reported in this strain (15). In order to genetically label the plasmid, kanamycin-resistant *Tn611* was used. Transfer of the mercury marker was selected in a strain of *M. smegmatis* harboring the thermosensitive and *Tn611*-containing plasmid pCG79 (9). This experiment was carried out at 32°C. In a second mating experiment, this transconjugant was used as the donor, and streptomycin-resistant *M. smegmatis* JAZ3 was used as the recipient (Fig. 1). Cointegration formation between pCG79 and the *M. fortuitum* plasmid, mediated by *Tn611*, was selected by plating of cells on kanamycin and streptomycin at 42°C. Several candidates were obtained.

![FIG. 1. Scheme of mating experiments carried out in order to label pJAZ38, pJAZ38 was first introduced to *M. smegmatis* harboring pCG79, the resulting transconjugant was then used as the donor in a second experiment, selecting for transposition of *Tn611* into pJAZ38. The plasmid obtained, pJAZ14, is shown with the insertion sequences depicted as triangles.](http://jb.asm.org/)

---

**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> XL1</td>
<td>Tc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22</td>
</tr>
<tr>
<td><em>M. fortuitum</em> 138</td>
<td>Hg&lt;sup&gt;b&lt;/sup&gt;, harbors pJAZ38</td>
<td>14</td>
</tr>
<tr>
<td><em>M. fortuitum</em> FC1</td>
<td>Environmental isolate</td>
<td>U.Z. Cult. Col.</td>
</tr>
<tr>
<td><em>M. fortuitum</em> 162</td>
<td>Clinical isolate</td>
<td>U.Z. Cult. Col.</td>
</tr>
<tr>
<td><em>M. fortuitum</em> 557</td>
<td>Clinical isolate</td>
<td>U.Z. Cult. Col.</td>
</tr>
<tr>
<td><em>M. smegmatis</em> mc&lt;sup&gt;155&lt;/sup&gt;</td>
<td>Competent for electroporation</td>
<td>23</td>
</tr>
<tr>
<td><em>M. smegmatis</em> EZ10</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td><em>M. smegmatis</em> JAZ3</td>
<td>mc&lt;sup&gt;155&lt;/sup&gt; mutant, Sm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pHP450</td>
<td>Sm&lt;sup&gt;r&lt;/sup&gt; Sp&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19</td>
</tr>
<tr>
<td>pB4</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt; derivative of pAL5000</td>
<td>21</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap&lt;sup&gt;c&lt;/sup&gt; lac&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28</td>
</tr>
<tr>
<td>pUC19-Km</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt; lac&lt;sup&gt;a&lt;/sup&gt;</td>
<td>U.Z. Cult. Col.</td>
</tr>
<tr>
<td>pJAZ38</td>
<td>Plasmid found in <em>M. fortuitum</em> 138</td>
<td>This study</td>
</tr>
<tr>
<td>pCG79</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt; ts Tn611</td>
<td>9</td>
</tr>
<tr>
<td>pJAZ14</td>
<td>pCG79-labelled pJAZ38</td>
<td>This study</td>
</tr>
<tr>
<td>pJAZ30</td>
<td>pJAZ38 and pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pJAZ42</td>
<td>pJAZ40 and Sp-Sm cassette</td>
<td>This study</td>
</tr>
<tr>
<td>pJAZ56</td>
<td>4-kb BamHI fragment of pJAZ40 in pUC-Km</td>
<td>This study</td>
</tr>
<tr>
<td>pJAZ57</td>
<td>PvuII subclone of pJAZ40 in pUC-Km</td>
<td>This study</td>
</tr>
<tr>
<td>pJAZ58</td>
<td>PvuII subclone of pJAZ40 in pUC-Km</td>
<td>This study</td>
</tr>
<tr>
<td>pJAZ59</td>
<td>PvuII subclone of pJAZ40 in pUC-Km</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ap, ampicillin; Sm, streptomycin; Sp, spectinomycin; Km, kanamycin; Tc, tetracycline; Hg, mercuric chloride; ts, thermosensitive replication; lac<sup>a</sup>, lac, lactose positive.

<sup>b</sup> U.Z. Cult. Col., University of Zaragoza Culture Collection.
Cointegration formation mediated by Tn611. Hybridization of total DNA from these candidates with insertion sequence IS6100 as a probe showed that 3 of 10 contained an extra copy of the IS as expected if transposition mediated by IS6100 had occurred. Electrophoresis of total DNA from the transposition candidates in E. coli resulted in the recovery of a recombinant plasmid, formed by cointegration between pCG79 and an extra fragment with a size of 16.9 kb. Restriction analysis of these three candidates showed them to be identical, while the seven other colonies analyzed were found not to contain an extra copy of IS6100. The cointegrate plasmid was named pJAZ14 (Fig. 1 and 2).

Replicative transposition, like that occurring on transposition of Tn611, results in the generation of an extra copy of IS6100 (0.8 kb) and duplication of a direct repeat (DR) sequence (reference 16 and our unpublished results). Partial sequencing of plasmid pJAZ14 was carried out of the flanking regions of the transposition insertion site in order to confirm the presence of the DR sequences and hence the fact that transposition had occurred. An 8-bp DR sequence, CTGCGCTC, was found flanking the IS, as expected as a result of transposition. Blast searches of the flanking regions of the transposon insertion site did not reveal significant homology with anything.

Origin of the M. fortuitum plasmid pJAZ38. Hybridization was carried out with total undigested DNA from donors and recipients used in the previous mating experiments in order to confirm that the labeled DNA pertained to M. fortuitum and was not present in M. smegmatis. The 2.1-kb EcoRI fragment was used as a probe (Fig. 2). Figure 3 shows the presence of extrachromosomal DNA which is present in M. fortuitum and not M. smegmatis. The 16.9-kb fragment present in pJAZ14 was cloned in a pUC19 HindIII site which cuts inside the IS element, resulting in the plasmid pJAZ40 (Fig. 2). This plasmid was used to confirm the size of the DNA insert and to compare its size with that of the element in the original M. fortuitum strain. Southern blot preparations of total DNA from M. fortuitum 138 were found to hybridize when the entire plasmid was used as a probe and, upon digestion prior to hybridization, gave banding patterns which corresponded exactly to the M. smegmatis mercury-resistant, transconjugant banding pattern, which was calculated to be 16.1 kb. The plasmid was named pJAZ38.

Replication and stability of pJAZ38 derivatives in M. smegmatis. In a further cloning experiment, the streptomycin-spectinomycin cassette was introduced to pJAZ40 by partial digestion with HindIII, resulting in the plasmid pJAZ42 (Fig. 2). Plasmid pJAZ42 was then introduced into M. smegmatis and was found to replicate at 32, 37, and 42°C. In order to determine the stability of pJAZ38 derivatives, pJAZ42 and pJAZ56 were transformed in M. smegmatis. The results of the study show that the plasmids are stably maintained in the absence of selection pressure. After 4 days (48 generations) of growth in antibiotic-free medium, only pJAZ56 suffers a loss of 1 order of magnitude (Fig. 4A). This lower stability of pJAZ56 compared with that of pJAZ42 may be due to the fact that while all of pJAZ38 is present in pJAZ42, pJAZ56 contains less than one-fourth the original plasmid, and it may therefore be lacking regions responsible for stability functions.

In compatibility studies, coculturing of the pJAZ38 derivative pJAZ42 (streptomycin resistant) with a pAL5000 derivative, pB4 (kanamycin resistant), showed that the number of cells which expressed both streptomycin and kanamycin was almost equal to the number of cells resistant to either antibiotic. These results indicate that these two plasmids coexisted in M. smegmatis for at least 48 generations (Fig. 4B).
Plasmid copy number. Relative plasmid copy numbers were determined by single-cell resistance to kanamycin. This method has been shown to work well for low- and medium-copy-number plasmids (25). The single-cell resistance for cells containing the constructs pJAZ56 and pB4 and for strain EP10 is shown in Fig. 5. The single-cell resistance for EP10 was set to 1, and it was maintained for pJAZ56, corresponding to a relative copy number of 1. In the case of pB4, there was an increase compared with EP10 corresponding to a relative copy number of 8. The results of these experiments correlated well with results obtained by hybridization experiments (data not shown).
Sequencing of the replication region. pJAZ40 was digested with BamHI, and the fragments obtained were cloned in pUC-Km; likewise, all of the fragments obtained on EcoRI digestion of pJAZ40 were cloned. These subclones were then electroporated in M. smegmatis mc2155, and a 6-kb EcoRI fragment was found to replicate, as was an internal 4-kb BamHI fragment (Fig. 6). Smaller subclones of pJAZ56 obtained on PvuII digestion were found not to replicate. These subclones were sequenced, and homology with two previous described mycobacterial plasmids, pMSC262 and pLR7, was found. The entire sequence of pJAZ56 was obtained (4 kb), and the region responsible for replication was defined (Fig. 7). Homology studies of this region of approximately 1.1 kb showed the region had 61% identity with both pLR7 and pMSC262 replicons at the DNA level, while at the amino acid level, homology studies revealed 53% identity and 66% similarity with only pLR7. Figure 7 shows 2.4 kb of the sequence obtained from pJAZ56, including the deduced amino acid sequence of the region responsible for replication. A comparison of the deduced amino acid sequence of pJAZ38 with that of pLR7 can be seen in Fig. 8.

Detection of related plasmids in other M. fortuitum strains. Total DNA from a further 32 strains of M. fortuitum of both clinical and environmental origin was analyzed by hybridization with pJAZ38 as the probe, and homologous plasmids were detected in three strains. Two of these strains were clinical isolates (M. fortuitum 162 and 557), while the third was environmental in origin (M. fortuitum FCI). Upon digestion of the DNA and rehybridization with the same probe, different restriction patterns were obtained. This indicates the presence of homologous but nonidentical plasmids throughout the mycobacteria.

DISCUSSION

We have described the genetic labelling of a cryptic mycobacterial plasmid, pJAZ38, by transposition of Tn611 into the M. fortuitum plasmid in a two-step mating experiment. The method used was a modified version of that described for the labeling of gram-negative cryptic plasmids by Tn3 replicative transposition (24). This method could be of use for the labeling of nonconjugative cryptic mycobacterial plasmids with Tn611 and the thermosensitivity of pCG79, where the vector is introduced by electroporation. Cointegrate plasmids could be transformed to E. coli, selecting for kanamycin resistance, thereby allowing further studies with E. coli.

Three plasmids have been reported in the M. fortuitum donor strain 138 (15). Following our detection of conjugative transfer of resistance to mercuric chloride to M. smegmatis, we failed to detect the presence of plasmids in agarose gels. We therefore set about the genetic labeling of the plasmid, and this resulted in the isolation of pJAZ38, which was found to be neither conjugative nor resistant to mercuric chloride. From
FIG. 7. Complete nucleotide sequence of the pJAZ38 replication region. The putative rep gene is indicated by the deduced amino acid sequence below the DNA sequence. Pvu II sites are shown in boldface.

---

**XhoI**

CTCGAG: AAGCCGCGGCTGCTCCGTGCGAGAGCGAGGCTCA

TGACGCGAACAACGCACTCCCTACCCCTCACTGCTGCTCAATGAGGATCTGAGACCCGGCTCAGTAAGCAGGCTACGCGCTCCCGCTCT

GTCGGACACCCCGTCTCTTCTCTCCATCCGAGCGCCGCGGCTACGCTCTTCGCCCGCTCCCGACCGCGCTACGCGCTCCCGCTCT

CAGCGCCGCGGCTACGTCCGAGCGCCGCGGCTACGCGCTCCCGCTCCCGACCGCGCTACGCGCTCCCGCTCT

ATGGCGCGGCTACGTCCGAGCGCCGCGGCTACGCGCTCCCGCTCCCGACCGCGCTACGCGCTCCCGCTCT

Pvu II sites are shown in boldface.
the experiments carried out to label pJAZ38, it would appear that although pJAZ38 is not conjugative, it is mobilizable, which suggests that another plasmid was responsible for the conjugative transfer and comobilization of pJAZ38.

The plasmid pJAZ38 was found to show homology with the *M. avium* plasmid pLR7 and the *M. scrofulaceum* plasmid pMSC262 at the DNA level. At the amino acid level, homology was obtained only with plasmid pLR7. The report of the sequence of the origin of replication of the *M. avium* plasmid pLR7 shows disagreement with that of the homologous plasmid pMSC262, and the authors suggest that in one of the cases, the wrong open reading frame was chosen (2).

The DNA homology between the *M. scrofulaceum* plasmid pMSC262, the *M. avium* plasmid pLR7, and the *M. fortuitum* plasmid pJAZ38, along with the similar sizes of approximately 16 kb, suggests that these plasmids are related, although less homology was seen outside the replication region. While there was 61% homology with both of the other plasmids in the replication region, the immediate upstream and downstream regions gave scores of less than 50% and approximately 40%, respectively. This would suggest that these are different, although related plasmids. Another important difference is the host range of pJAZ38, which can replicate in *M. smegmatis* mc²155 while the other two plasmids cannot.

The elevated numbers of *M. avium* isolates from AIDS patients and the detection of pLR7-related plasmids in these...
strains have led some authors to suggest a possible role of the pLR7 family of plasmids in infection and colonization of immunocompromised hosts (3). Furthermore, the presence of homologous plasmids throughout the genus Mycobacterium would seem to suggest that horizontal gene transfer occurs, although this fact had not been proved. The method by which pJAZ38 was recovered by mobilization due to the presence of a putative coexisting conjugative plasmid supports the theory of such horizontal transfer as would occur in nature. Interestingly, in the environmental isolate of M. fortuitum FC1 from which the sulfonamide-resistant transposon Tn610 was isolated, the presence of pLR7-like plasmids was detected. Homologous plasmids were also detected in two clinical isolates. Further evidence of gene transfer involving mycobacteria in nature would be the detection of IS6100, originally isolated from M. fortuitum FC1 in both Flavobacterium species and Pseudomonas species (13), and the fact that the sulfonamide-resistant element isolated from M. fortuitum was shown to be identical to sulfonamide resistance genes from E. coli (16).

The isolation of a new mycobacterial replicon is of particular interest, because to date the majority of vectors available for use in mycobacteria are based on pAL5000 or nonmycobacterial, broad-host-range vectors such as RSF1010, which have been found to replicate in mycobacteria (7). The plasmid described here, pJAZ38, was found to replicate in M. smegmatis mc²155, unlike related plasmids pLR7 and pMSC262. Moreover, the fact that the new replicon is compatible with pAL5000 makes it very important for potential use in complementation studies. pJAZ38 was also found to be stably maintained in mycobacteria in the absence of selection pressure. All of these findings make pJAZ38 a good candidate for further genetic studies with mycobacteria. Further studies are currently under way in our laboratory to identify the region responsible for mobilization of pJAZ38.

ACKNOWLEDGMENTS

We thank Rafael Gómez-Lus for interest in this work. We are indebted to E. Gormley, F. de la Cruz, J. M. García-Lobo, and B. Gicquel for advice and helpful discussions and Ian Ross for help with the final preparation of the manuscript.

This work was supported by the European Union Programme BIOMED 2 Project (PL95-1241) and the Spanish Ministry of Health (FIS 97/0042).

REFERENCES